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Work done in previous years has clearly documented that the 90kD glycoprotein is a clinically important tumor-associated antigen (TAA) expressed by breast cancer cells. The assay developed to detect its presence in serum provides valuable information in identifying breast cancer patients post-operatively who are at high risk of developing recurrent disease. We have purified the 90kD TAA to near homogeneity and elucidated many of its physico-chemical properties. The 90kd TAA clearly is a heat stable glycoprotein which has affinity for various lectins, e.g., wheat germ agglutinin, lentil lectin. Its antigenic activity is not affected by treatment with mixed glycosidases but affected by treatment with proteases. The purified TAA did not exihibit any immunoreactivity in Western blot with murine monoclonal antibodies developed by other investigators, and that have been shown to react with other macromolecules expressed by breast cancer cells. The purification schema developed to purify the 90kD TAA is such that it will be applied to isolate and purify other antigens recognized in Western blot after reacting with breast cancer patients serum or supernates of lymphoblastoid clones. Work is in progress to sequence the purified 90kD TAA and clone the gene that encodes the antigen.

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Rishab K. Gupta 10-16-97

PI - Signature Date

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INTRODUCTION

A recent report on the 20 years follow-up by the Breast Cancer Detection Demonstration Project (BCDDP) denotes that there is a decrease in the rate of advanced breast cancer (1). This is attributable to early detection of the disease. Late last year, the American Cancer Society, the National Cancer Institute, and the Center for Disease Control and Prevention reported a decline in breast cancer mortality rates in the United States. This decrease was attributed to both early detection and improved treatment modalities(2, 3). Thus, breast cancer is no longer the leading cancer of women (4). However, it still remains an issue of utmost importance, because it accounts for 32 percent of malignacies in females (5). It has been determined that the incidence of breast cancer has been increasing steadily since the 1960s at a rate of 1 to 2 percent per year in the United States. For women between the ages of 15 and 54 years, breast cancer is the most common cause of cancer related death (6). A number of tumor markers have been developed for early detection of breast cancer (7-10); however, their impact on survival and quality of life remains questionable (11). We believe that the limited success achieved by various investigators in developing in vitro correlations of human tumor immunity that are prognostically useful was largely due to unavailability of well-characterized immune reagents. We realize that the problem of tumor-host interaction is extremely complex; however, we are convinced of the importance of tumor immunology in prognostication of the clinical course of breast cancer in humans. The key to our understanding more fully the problem is to work towards determining the clinical significance of the defined and refined human tumor antigens, and to determining the immune responses of the host to these antigens.

During the previous years we have documented that breast cancer cells express tumor associated antigens that are immunogenic in the patients. As a result breast cancer patients have antibodies that can react with their own as well as already established breast cancer cell lines. Specificity of these antibodies has been determined by absorption studies and differential reactivity to normal and breast tumor cell extracts. Thus, upon appearance of the antigens due to subclinical tumor growth, humoral antibodies react with these antigens and result in the formation of TAA-specific immune complexes (IC). As a result, tumor antigen specific IC occur in the serum before free antigen could persist (12). We developed the tumor antigen specific IC detection technology which circumvented the problems encountered in earlier studies that applied the antigen non-specific immune complex assay techniques as the tumor markers (13). Quantification of TAA-specific IC by a sensitive method has served as a reliable marker for prognosis of breast cancer. Furthermore, detection of TAA-specific IC by the methods which we have developed gives an amplification effect and makes the assay more sensitive to determine the presence of TAA in a given sample (14).

In recent years, there has been an interest and trend to utilize aberrantly altered and/or overexpressed molecules as potential targets for therapy including immunotherapy, particularly when the molecules are antigenically distinct and can elicit immune responses in the host. The 90kD glycoprotein that has been extensively investigated with respect to its presence in

circulation of breast cancer patients in the form of immune complexes is apparently an ideal molecule for this purpose. However, before it can be proposed or utilized for this purpose, it is absolutely necessary to purify the antigen to homogeneity and characterized as much as possible with respect to its physico-chemical nature and composition. It would be safer and appealing to work with a molecule of known properties and function to utilize it for any therapeutic purpose. Therefore, in addition to the approved Statement of Work (SOW), we devoted considerable efforts to further characterize the purified 90kD glycoprotein tumor-associated antigen (TAA). To purify the antigen from urine of cancer patients, procedures were optimized and standardized that included centrifugation, ultrafiltration, and other biochemical approaches as outlined in the subsequent sections. Also, we have initiated work to clone the gene that encodes for the antigen recognized by a patients serum as the source of antibody in the Western blot technique.

BODY

EXPERIMENTAL METHODS:

Since urine samples of several breast cancer patients were positive for the glycoprotein TAA, we obtained 24 hour urine collections from the positive patients. The pool was used as the starting material to purify the antigen.

PURIFICATION PROCEDURE:

Collection of urine was done in Tris-HCl (pH 8.3) buffer supplemented with sodium azide to give a final concentration of 0.1 M Tris and 0.02% azide, and processed as outlined in Figure 1 of the Appendix. A total volume of 14.4 liters of urine was centrifuged at 6,000 x g for 10 min and filtered through #1 Whatman filter paper. The clarified sample was concentrated 100-fold using a hollow-fiber concentrator (10kD exclusion limit, Amicon Corporation, Danvers, Mass.) and then subjected to pressure ultrafiltration through a PM-10 membrane (10kD cutoff limit, Amicon Corporation). The concentrate was centrifuged at 800 x g for 10 min. The clearified supernate was subjected to gel filtration chromatography through a calibrated Sephacryl S-200 column (1.5 X 100 cm). Sodium phosphate (0.025 M, pH 7.2) buffer supplemented with 0.15 M NaCl and 0.02% sodium azide (PBS) was used as the eluent at a flow rate of 20 ml per hour. Five ml fraction per tube were collected and the elution of the protein was monitored at 280_{nm} (Figure 2).

Fractions exhibiting greater than 0.1 OD at 280nm were pooled, and the pooled peaks were concentrated back to the volume initially applied to the column and analyzed for protein concentration and the TAA activity. Protein concentration in each pool was assessed by the method of Lowery et al (15), and the TAA activity was determined by a capture ELISA using murine monoclonal antibody AD1-40F4 as the catcher.

ABSORPTION WITH RABBIT ANTI-HUMAN Ig IMMUNOBEADS:

The antigenic fraction (peak I) was treated with rabbit anti-human immunoglobulin antibodies that were immobilized to agarose beads (Immunobeads - BioRadiation Laboratories, Richmond, CA). Five ml of the peak was added to 5 ml packed volume of the immunobeads, and the mixture was incubated at room temperature with continuous end-over-end mixing on a circular rotator for one hr. The absorbed antigen was recovered by centrifugation at 800 x g for 10 min, and subjected to reduction and alkylation.

REDUCTION AND ALKYLATION:

The immunoaffinity (immobilized human anti-Ig) purified TAA was reduced with dithiothreitol in the presence of 6M guanidine hydrochloride and alkylated with iodoacetic acid at pH 8.5 according to the procedure described by Ozols (16), and the products were separated by Sephacryl S-200 column (1.0 x 60 cm) chromatography using 1 M propionic acid as eluent (Figure 3).

POLYACRYLAMIDE GEL ELECTROPHORESIS:

One dimensional polyacrylamide gel electrophoresis under reducing conditions was performed according to the procedure described by Laemmli (17) using a 4 to 15% gradient gel.

Two dimensional gel electrophoresis was performed using the isoelectric focusing gel which contained acrylamide (4% T and 4% C), 9M urea, 9.2mM CHAPS, 0.4% NP-40 and 5% ampholyte (60%, 3-10; 20%, 5-7; 20%, 6-8) in the first direction. The sample was electrofocused at 600 V for 20 hrs. The second dimension electrophoresis was a mass separation through SDS-acrylamide (10 to 20% T and 2.6% C) gel at 30 V and 12C for 4 hrs. Protein spots were visualized by silver staining.

REVERSE PHASE HPLC:

Reverse phase HPLC analysis of the samples was performed using a Delta-Pak C4 (5um particle with 300 A pore size) column and 5% to 95% acetonitrile linear gradient in 0.1% trifluoroacetic acid. The separation was performed in H-P model 1090M HPLC system. The identity of peaks were determined on the basis of retention time.

WESTERN BLOT:

Western blotting technique (18) was used to determine immunoreactivity of various monoclonal antibodies to the purified 90kD TAA. Two microgram protein of the purified 90 kD glycoprotein per lane was subjected to SDS-PAGE and electroblotted to nitrocellulose membrane. After washing and blocking with 5% non-fat milk, the membrane was cut into 5mm wide strips. The strips were reacted with monoclonal antibodies at 1:100 dilution for ascites or

1:25 dilution for hybridoma culture supernates at 4C for 12 hrs. Goat anti-mouse Ig conjugated to alkaline phosphatase (Sigma Chemical Co., Saint Louis, MO) at 1:500 dilution and NBT/BCIP were used invariably to determine reaction of the murine monoclonal antibody.

RESULTS:

PURITY OF 90kD TAA:

The glycoprotein TAA purified by the procedures outlined in Figure 1 appeared to be comprised of several bands by SDS-PAGE analysis (Figure 4, lane 2). Therefore, peak I of Figure 2 was subjected to reduction and alkylation, and the reduced product separated by Sephacryl S-200 column (1.0 x 60 cm) chromatography. The second peak (Figure 3) of the reduced and alkylated material clearly showed a single band in SDS-PAGE after silver staining (Figure 4, lane 4) and this band was reactive with the murine monoclonal antibody, AD1-40F4, (Figure 4, lane 6) in Western blot. The overall results of 90kD TAA purification achieved by the steps outlined in Figure 1 are summarized in Table 1. Clearly, this procedure allowed 1,000-fold purification with 13.9% yield of the antigen from the starting material.

Comparison of densitometric scans of the silver stained gels using a gel scanner (Shimadzu, Model CS9000U) of the starting material (100-fold concentrated urine) and the purified 90kD TAA revealed that while 90kD TAA was only 0.4% of total protein in the starting material, this component was greater than 89.4% of total protein in the most purified fraction Figure 5). This level of purity (about 90%) was confirmed by two dimensional gel-electrophoresis where only one spot could be revealed by silver staining.

To further determine the level of purity and to compare the protein profile of final preparation of 90 kD subunit of U-TAA with that of starting material (100-fold concentrated urine), the samples were subjected to reverse phase HPLC analysis using Delta-Pak C4 (5um particle with 300 A pore size) column and 5% to 95% acetonitrile linear gradient in 0.1% trifluoroacetic acid. The separation was performed in H-P model 1090M HPLC system. The starting material (100-fold concentrated urine) was reduced with DTT and alkylated with iodoacetic acid as described above. Ten microliter sample was injected in each case. Figure 6A illustrates the 214nm absorbing profile of the starting material. Clearly at least 17 peaks are visible after 20 min of retention time. On the contrary, only a single peak with a slight shoulder on the left side with a retention time of about 36 min was seen in the profile of the purified 100 kD subunit of U-TAA (Figure 6B). Only a very minor peak was detected at this position in the profile of the starting material (Figure 6A).

REACTIVITY OF VARIOUS MURINE MONOCLONAL ANTIBODIES TO TAA IN WESTERN BLOT:

The 90kD TAA is expressed by various solid neoplasms including breast carcinoma and

melanoma. We realize that murine monoclonal antibodies that react with tumor cells or their lysate have been developed using tumor cells or partially purified tumor antigen fractions as the immunogen. Therefore, a possibility existed that the 90kD TAA which we purified could be an antigen recognized by the already available antibodies. To rule out this possibility, we procured several murine monoclonal antibodies either directly from the investigators. These murine monoclonal antibodies were used in Western blot to determine their reactivity with purified 90kD TAA (material depicted in Figure 6B). Two microgram protein of the 90kD TAA per lane was subjected to SDS-PAGE and electroblotted onto nitrocellulose membrane. After washing and blocking with 5% non-fat milk, the membrane was cut into 5mm wide strips. The strips were reacted with monoclonal antibodies at 1:100 dilution for ascites or 1:25 dilution for hybridoma culture supernates at 4C for 12 hrs. Goat anti-mouse Ig conjugated to alkaline phosphatase (Sigma Chemical Co.) at 1:500 dilution was used invariably to determine reaction of the murine monoclonal antibody.

None of the seven murine monoclonal antibodies to either melanoma or breast cancer cells developed by various investigators reacted with the 90kD TAA; however, under similar experimental conditions, AD1-40F4 (at 1:500 dilution of ascites) -- an IgM murine monoclonal antibody developed by us using the TAA as immunogen -- showed positive reaction (Figure 7). These results denote that 90kD TAA does not bears any epitope which is recognizable by any of the seven murine monoclonal antibodies we have tested thus far.

DISCUSSION:

We devoted considerable efforts towards continuously growing the LCL-4 lymphoblastoid cell line to accumulate the secreted antibody; however, the amount of immunoreactive IgM antibody that could be isolated by cold precipitation was adequate only to be used in a limited number of ELISA or Western blots. Also, we have had limited amount of IgG purified from patient's serum. Therefore, we were unable to use the immunoaffinity techniques to isolate the high molecular weight antigen or the 53kD antigen from breast cancer cell extract in a manner we thought last year. We will now devote our efforts to apply the same approaches as we have used during this year for 90kD TAA to isolate and purify the above mentioned antigens.

During this report period, we placed emphasis on purifying the 90kD TAA because our previous investigations, as reported last year, have deocumented the clinical importance of this antigen. These investigations allowed us to purify the 90kD TAA to near homogeneity and to characterize the purified antigen with respect to its immunological and physico-chemical properties. The 90kd TAA clearly is a heat stable glycoprotein which has affinity for various lectins, e.g., wheat germ agglutinin, lentil lectin. Its antigenic activity is not affected by treatment with mixed glycosidases but affected by treatment with proteases. Since the purity of the antigen is high, we are moving forward to determine its amino acid composition and sequence. We believe that such information will be very useful in generating molecular probes to clone the gene encoding for the antigen.

Up to this point of the grant period we accomplished many of the tasks of the SOW. We have not only determined the clinical utility of the TAA-specific immune complexes in breast cancer patients, but also have characterized the antigen to near homogeneity. We are continuing our efforts in establishing lymphoblastoid cell lines that secrete IgG antibodies to antigens, particularly the 53kD, expressed by breast cancer cells. Technology developed for purification of the 90kD TAA should be applicable to the purification of the 53kD antigen.

CONCLUSIONS

Work done, in previous years has clearly documented that the 90kD glycoprotein is a clinically important antigen expressed by breast cancer cells. The assay developed to detect its presence in serum, though not 100% accurate, provides valuable information in identifying breast cancer patients who are at high risk of developing recurrent disease. Since the antigen is immunogenic in breast cancer patients as determined by the presence of antibodies to the antigen, it is possible that this antigen may be potentially useful for therapeutic purposes. However, before embarking on that aspect, it is necessary to characterize the antigen. We have purified the 90kD TAA to near homogeneity and elucidated many of its physico-chemical properties. The 90kd TAA clearly is a heat stable glycoprotein which has affinity for various lectins, e.g., wheat germ agglutinin, lentil lectin. Its antigenic activity is not affected by treatment with mixed glycosidases but affected by treatment with proteases. The purification schema developed to purify the 90kD TAA is such that it will be applied to isolate and purify the 53kD band visible in Western blot after reacting with a breast cancer patients serum in our previous studies.

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TABLE 1

YIELD AND FOLD-PURIFICATION OF 90kDa SUBUNIT OF TAA

Purification step	Total volume (ml)	Protein conc. (mg/ml)	Total protein (mg)	Antigenic activity (units/mg)*	Total antigen (units)	Purification (fold)	Yield (%)
100x concentrated urine	144	19.98	2877	25	71,925	1	100
Sephacryl S-200 peak-I	140	0.21	29.4	1190	35,000	47.6	48.7
Sephacryl S-200 peak-II after reduction and alkylation	10	0.04	0.4	25000	10,000	1,000	13.9

* One unit of antigenic activity is defined as the minimum amount of protein required to cause 50% inhibition of binding between TAA and AD1-40F4.

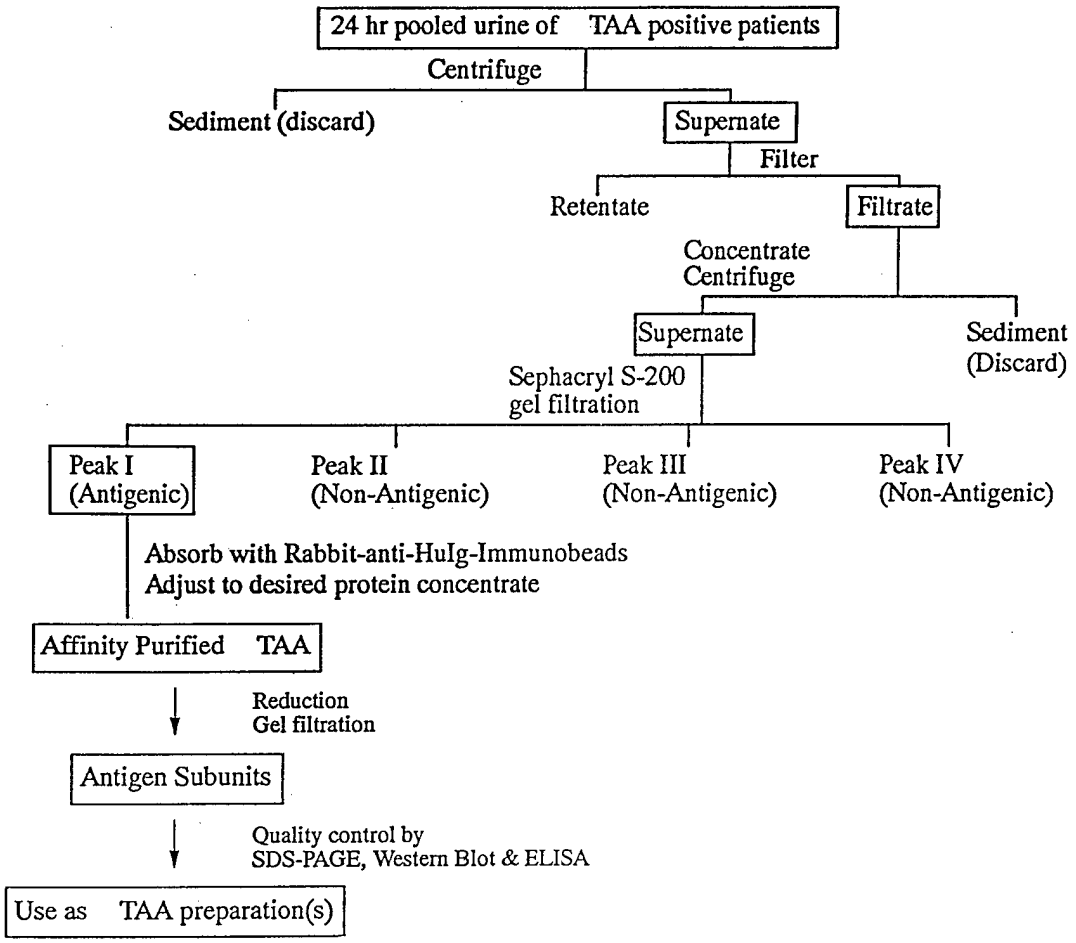

FIGURE 1: Purification protocol for 90kD tumor-associated antigen (TAA) from positive breast cancer patients. Details are given on page 6 of the text.

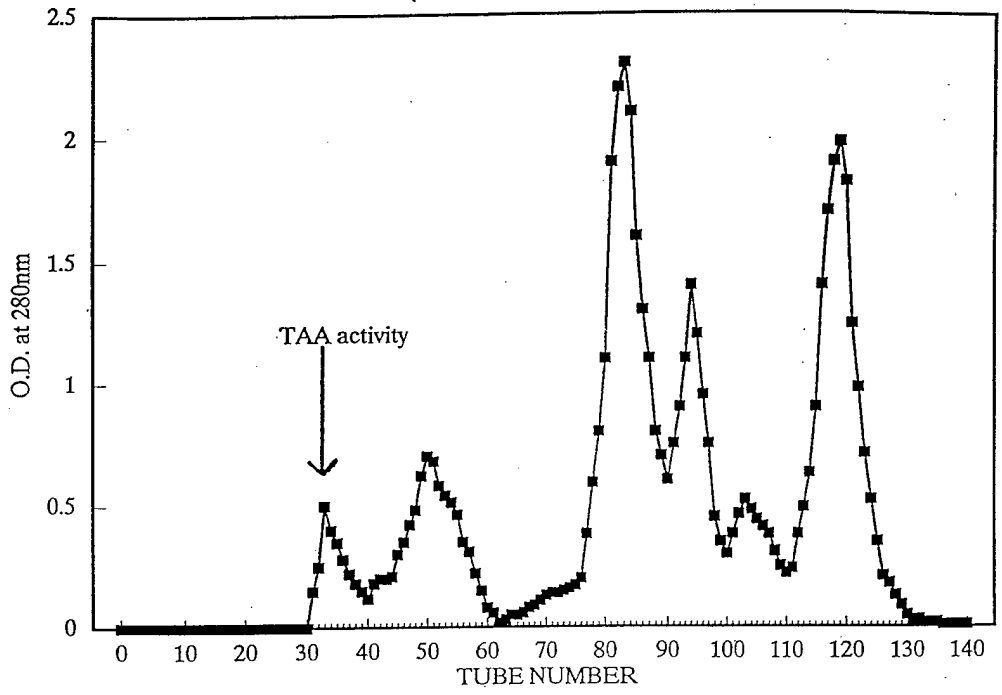

FIGURE 2: Elution profile of 100-fold concentrated urine from breast cancer patients from a Sephacryl S-200 column (1.5 x 100 cm). Phosphate buffer (0.025M) supplemented with 0.15M NaCl and 0.02% sodium azide was used as an eluent at a flow rate of 20 ml per hr. Five ml fractions per tube were collected and elution of protein was monitored at 280_{nm}. Tubes with OD_{280nm} of >0.1 under each peak were pooled, concentrated to the original volume and assessed for TAA activity against the murine monoclonal antibody, AD1-40F4, in ELISA.

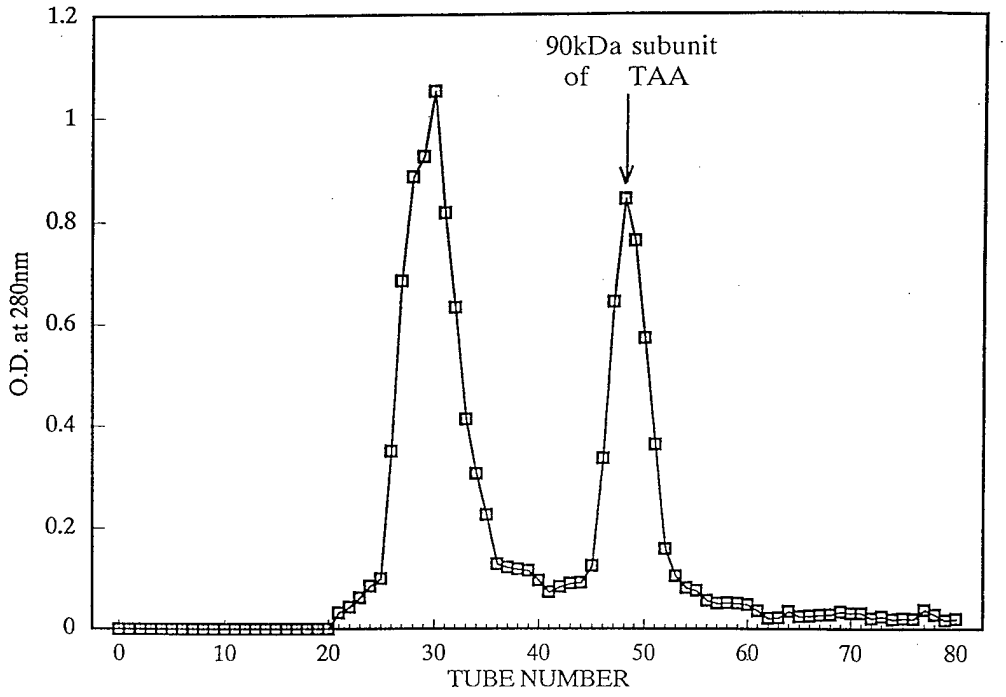

FIGURE 3: Elution profile from Sephacryl S-200 column (1.0 x 60 cm) of reduced and alkylated TAA preparation. 1.0M propionic acid was used as the eluent, and 2 ml fractions per tube were collected. Protein elution profile was monitored at 280_{nm}. Tubes under peak with >0.25 OD were pooled, dialyzed against PBS at pH 7.2, and tested for TAA activity by competitive inhibition in ELISA using AD1-40F4 as the target antibody.

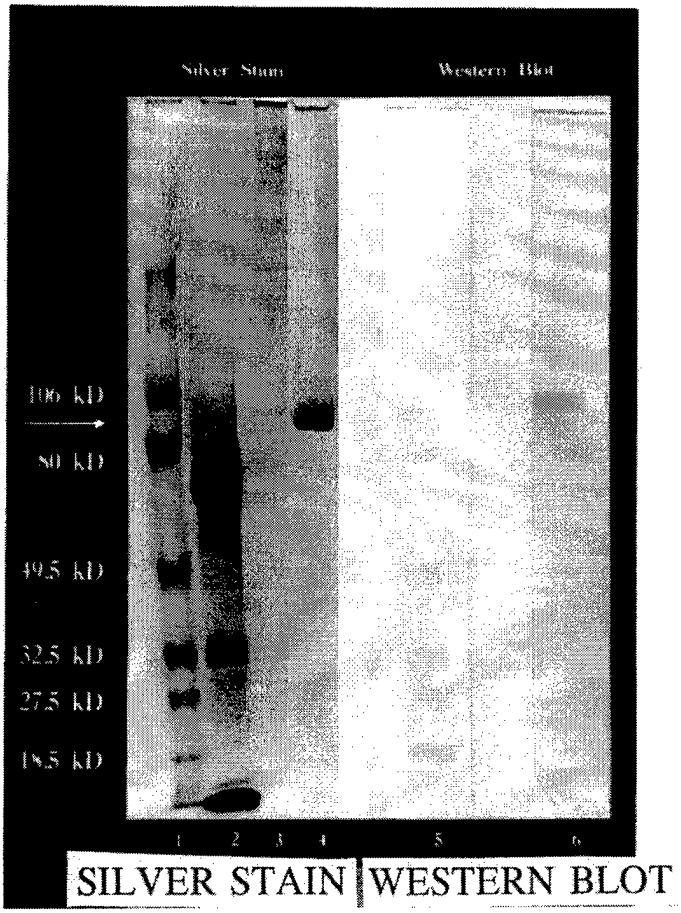

FIGURE 4: SDS-PAGE (4-15% gradient) and Western blot analysis under reducing conditions (2-mercaptoethanol, 5%) using Tris-glycine buffer at ph 8.3.

SDS-PAGE RESULTS:

Lane 1: Molecular weight markers.
Lane 2: 100x concentrated breast urine.
Lane 3: Sephacryl S-200 peak-I (Figure 2).
Lane 4: Sephacryl S-200 peak-II of reduced and alkylated TAA representing the murine monoclonal antibody immunoreactive 90kD subunit (Figure 3).

WESTERN BLOT RESULTS:

Lane 5: Molecular weight markers.
Lane 6: 90kD TAA (same material as in lane 4) reacted with the murine monoclonal antibody, AD1-40F4, after transfer to nitrocellulose membrane.

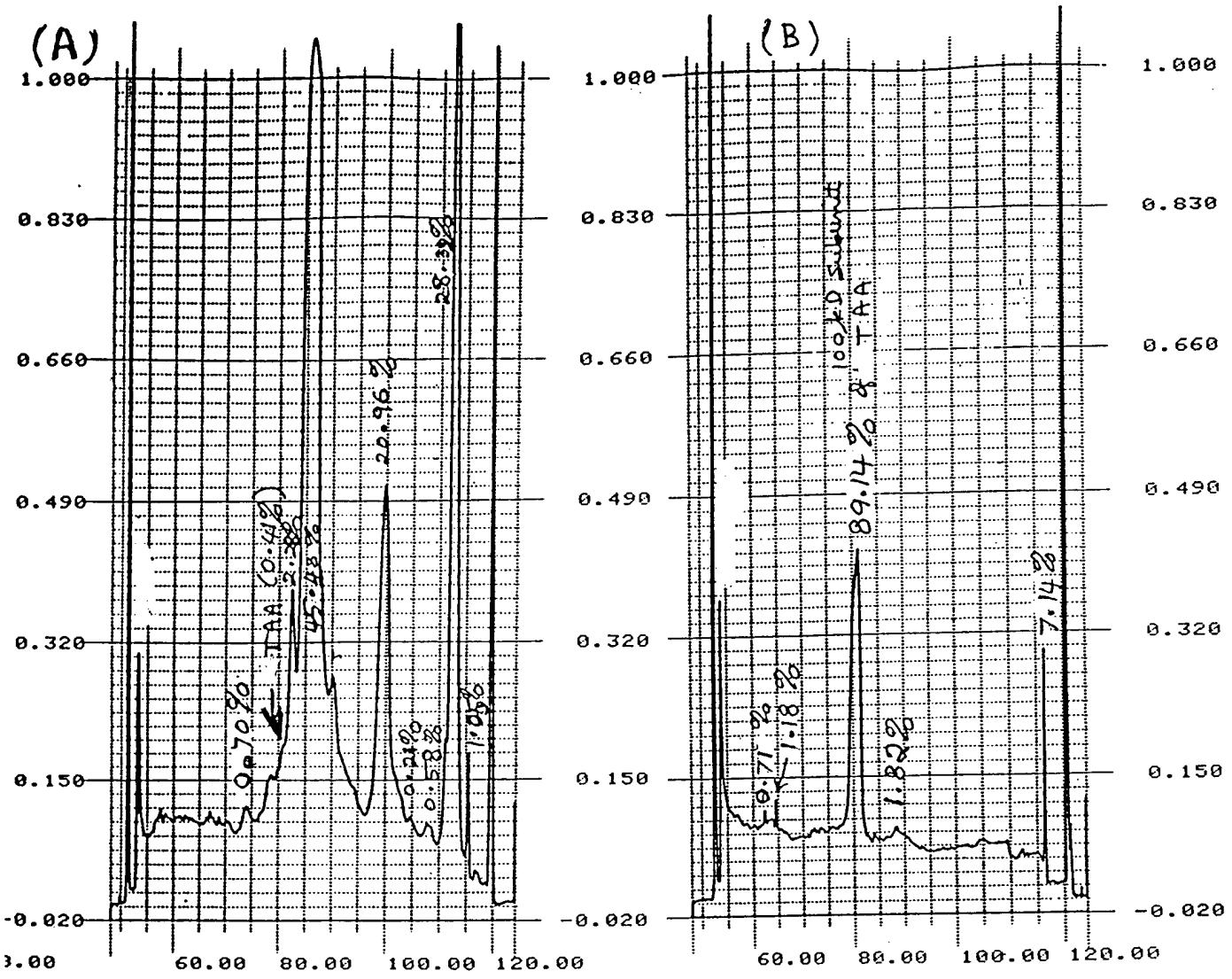

FIGURE 5: Densitometric scan of SDS-PAGE performed under reducing conditions. (A) Starting material (100x concentrated urine). (B) Purified 90kD TAA (Purity level: about 90%).

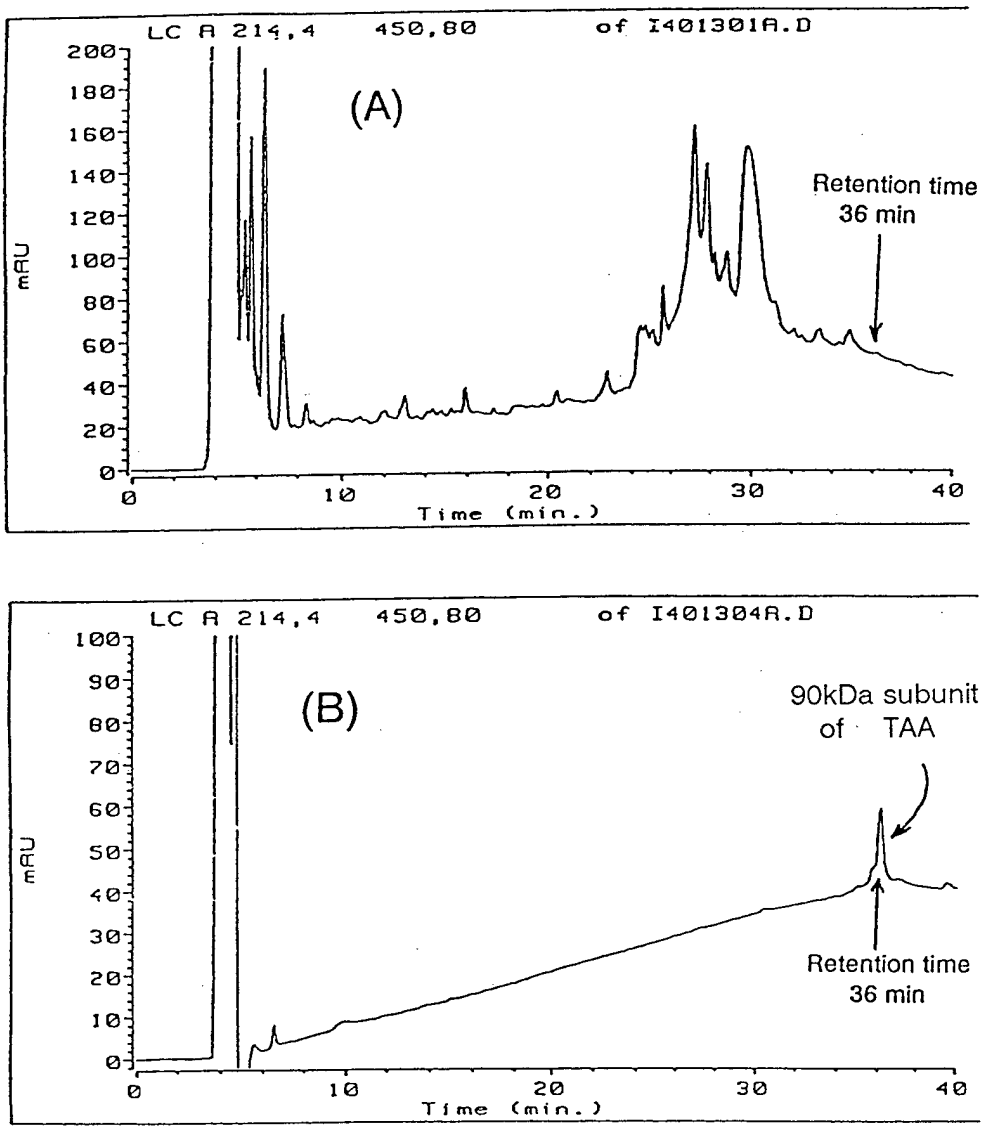

FIGURE 6: Reverse phase HPLC profiles of (A) starting material (100x concentrated urine, and (B) purified 90kD TAA using a Delta-Pack C4 column and 5-95% acetonitrile gradient in 0.1% trifluoroacetic acid.

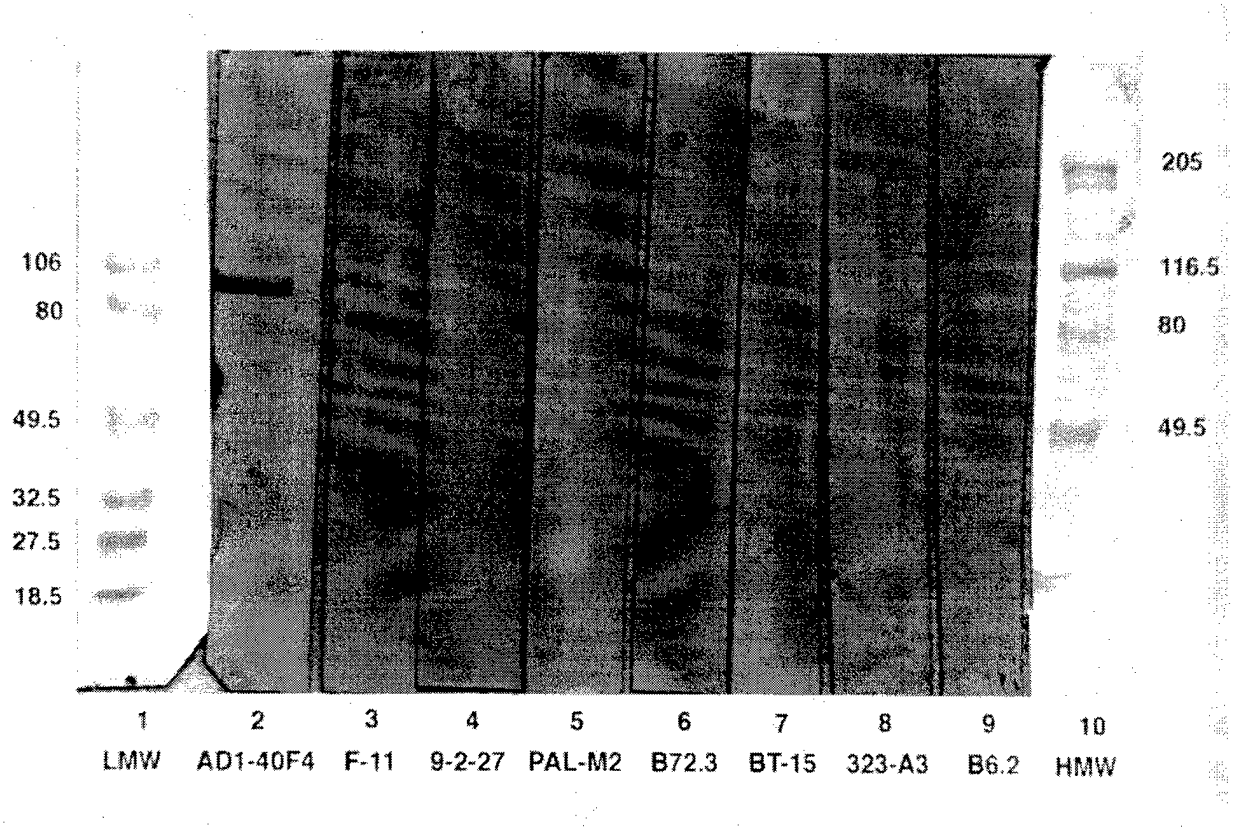

FIGURE 7: Immunoreactivity of 90kD TAA with various murine monoclonal antibodies in Western blot.

Lane #	Immunogen to develop MuMoAb	MuMoAb recognizes	Reference
1	Low molecular weight markers		
2	Glycoprotein TAA	90kD	(19)
3	Partially purified spent medium	75/77 & 100kD molecules	(20)
4	Melanoma cell extract	240kD	(21)
5	Melanoma cells	95-100kD	(22)
6	Breast cancer cells	TAG-72	(23)
7	Breast cancer cells	80-85kD	(24)
8	Breast cancer cells	43kD	(25)
9	Breast cancer cells	90kD	(26)
10	High molecular weight markers		